



Human astrovirus C-terminal nsP1a protein is involved in RNA replication

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Abstract

Human astrovirus nonstructural C-terminal nsP1a protein, which contains a hypervariable region (HVR) and colocalizes with the endoplasmic reticulum and viral RNA, has been suggested to be involved in the RNA replication process. Four viruses differing only in their C-terminal nsP1a protein, corresponding to HVR-derived genotypes IV, V, VI, and XII, were all able to replicate in CaCo-2 cells but displayed differences in their RNA replication and growth properties. Two overall patterns of replication were observed: types IV and V on one side, and types VI and XII on the other. The main detected differences were on the levels of antigenomic and subgenomic RNAs, being the latter significantly higher in types IV and V. Accordingly, quantification of viral RNA load in feces from children with gastroenteritis showed that HVR-derived genotypes IV and V occur in significantly higher numbers. In consequence, it may be concluded that the variability of the C-terminal nsP1a gene affects the virus replication phenotype.

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Human astrovirus (HAsV) is recognized as one of the leading causes of childhood gastroenteritis worldwide (Glass et al., 1996; Walter and Mitchell, 2003). These non-enveloped positive-strand RNA viruses belong to the family *Astroviridae*, which includes both mammalian and avian viruses. Their 6.8-kb polyadenylated genome contains three overlapping open reading frames (ORFs) (Jiang et al., 1993): ORF1a and ORF1b, which are linked by a translational ribosomal frameshifting and encode the viral protease and polymerase, respectively, and ORF2, which encodes the capsid precursor (Matsui and Greenberg, 2001).

Upon infection, two large nonstructural proteins are translated from the genomic RNA (nsP1a and nsP1a/1b), which mature by proteolysis giving rise to the viral proteins implicated in the transcription of a full-length negative-strand RNA molecule (antigenomic RNA). It is believed that this RNA molecule serves as a template for the transcription of both the new full-length 6.8-kb-genomic and the ORF2-containing 2.4-kb-subgenomic RNA molecules (Matsui et al., 2001). Translation of this subgenomic

RNA is used to produce large amounts of structural proteins for the efficient assembly of progeny viruses (Monroe et al., 1991). The precursor and mature products resulting from the HAsV nonstructural polyprotein processing have only been partially characterized, and some of the reported data are still conflicting (Geigenmüller et al., 2002; Gibson et al., 1998; Kiang and Matsui, 2002; Méndez et al., 2003; Willcocks et al., 1999). At present, it is believed that nsP1a and nsP1a/1b polyproteins are proteolytically processed by both cellular and viral proteases, giving rise to at least five products, which could be even further processed (see Fig. 3). Using antibodies against approximately 30% of nsP1a C-terminal region, different authors have identified products of 34, 20, 6.5, and 5.5 kDa (Méndez et al., 2003; Willcocks et al., 1999). In our laboratory, immunoprecipitation studies using an antibody against a synthetic peptide of the C-terminal nsP1a sequence (Guix et al., 2004a) led to the detection of five proteins in the range of 21–27 kDa, and at least one of them could be post-translationally modified by phosphorylation on its serine and threonine residues (Guix et al., 2004b).

Close to the C-terminus end of nsP1a polyprotein and contained within the putative C-terminal nsP1a protein, a

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hypervariable region has been identified by different authors (HVR, nucleotides 2363–2554 of HAsV1 ORF1a, accession no. L23513), but its biological significance remains unknown (Méndez-Toss et al., 2000; Oh and Schreier, 2001; Willcocks et al., 1994). Recent results obtained in our laboratory indicate that the C-terminal nsP1a protein, named in those studies nsP1a/4, colocalizes with the endoplasmic reticulum-derived membranes and with viral RNA, suggesting a potential role in the replication process (Guix et al., 2004b). Interestingly, within the HVR, Willcocks et al. (1994) described a 45-nt deletion, which was associated to the adaptation of HAsV to certain cell lines. Additionally, this HVR is contained in an immunoreactive epitope (Matsui et al., 1993). A nuclear localization signal (NLS) and a death domain (DD) have also been described upstream of the HVR (Guix et al., 2004a, 2004b; Jiang et al., 1993).

Since viral replicative functions are encoded by ORF1a and ORF1b, and since all complete HAsV genome sequences available at GenBank are highly conserved throughout all the nonstructural proteins with the exception of the C-terminal nsP1a HVR, it seems likely that this region could influence the viral RNA replication properties. This work aimed to understand the relationship between the high degree of genetic variability found in the C-terminal nsP1a protein and the efficiency in viral replication. Different mutants have been constructed and characterized with respect to growth in cell culture and RNA synthesis.

Results

Analysis of the genetic variability: association between HVR-derived genotypes and viral RNA load in fecal samples

To accurately define the extent of genetic variability within the HVR described in the C-terminal nsP1a protein, this region was sequenced from a collection of 46 HAsV isolates belonging to different serotypes. Many in-frame insertions and deletions (*indels*) were found within this region, as well as many nucleotide substitutions, without complete correlation with serotype information. Approximately 50% of nucleotide substitutions resulted in a change of amino acid, and only 9% of all the variable positions induced conservative amino acid substitutions. Based on

HVR nucleotide genetic variability, different C-terminal nsP1a protein HVR-derived genotypes were established, according to its *indel* pattern and the following definition: p-distance between all sequences within each genotype should be lower than 7% and between different genotypes should be higher than 7%. Using these criteria, a total of 15 HVR-derived genotypes were identified and named using roman numerals (manuscript in preparation). The analyzed HAsV isolates were classified into five HVR-derived genotypes (I, II, IV, V, VI). Most prevalent amino acid sequences within each genotype are presented in Fig. 1.

Information on the virus load present in stool suspensions could be obtained in 33 out of the 46 fecal samples by competitive RT-PCR (Fig. 1). An analysis of variance (ANOVA) of the number of genomes per gram of feces in samples belonging to different HVR-derived genotypes revealed that genotypes IV (11.96 ± 0.35) and V (11.92 ± 0.43) occurred in significantly higher numbers ($P < 0.05$) (Fig. 1). The time of collection, which could be relevant in terms of the viral load, should not influence the differences regarding virus titers observed between genotypes, since all samples had been randomly collected between days 1 and 3 after the onset of symptoms.

A definitive relationship between fecal viral load and HVR-derived genotype cannot be established from these data, since other genomic regions could also be responsible for the observed differences between them. However, the putative involvement of the nonstructural protein encoded by this region in viral RNA replication (Guix et al., 2004b), as well as its high degree of variability compared with the high degree of conservation for the rest of the region, could partially explain the differences in the titer found in feces between different HVR-derived genotypes.

Generation of recombinant astroviruses differing in their C-terminal nsP1a protein

In order to rule out the possibility that other parts of the genome could also participate in modulating the efficiency of RNA replication, three mutant astroviruses with different C-terminal nsP1a coding genes were generated using the HAsV1 reference strain full genome-length cDNA clone pAVIC (Geigenmüller et al., 1997). Three strains corresponding to different HVR-based genotypes (IV, V, and VI) were

Mean \pm SD of
Log₁₀ Genomes/gram (n)

HAsV-IV	--QQTNTSQKSHPELE-----AESQPLDLSQKK-----EKQPEHEQQAVKPIKPQKNEPQFYSQTYGK	11.96 \pm 0.35	(7) *
HAsV-V	-----KPCPEPE-----TEAQPLDLSQKK-----EKQPEQEQQVAKPTKSQKSEPPFYSQTYGK	11.92 \pm 0.43	(5) *
HAsV-II	-----KPCPEPESNKPALTEAQPLDLSQKK-----EKQTEHEQQMAKPVKHQKSEPPFYSQTYGK	10.80 \pm 0.67	(9)
HAsV-VI	VLTSSEKLPKPCPEPE-----AEAQPLDLSQKKAENPEKQPEYETQVVKP-KPQKSEPPFYSQTYGK	10.67 \pm 0.36	(7)
HAsV-I	-----KICPEPE-----AETQPLDLSQKK-----EKQPEHEQQVVKSTKPQKNEPQFYSQTYGK	10.45 \pm 0.24	(5)

Fig. 1. Amino acid alignment of the HVR (positions 768–823 for type IV, 768–816 for types V and I, 768–822 for type II, and 768–829 for type VI) within the C-terminal nsP1a protein. Most prevalent sequences within each type are presented, and the mean titers of viral genomes per gram of feces obtained from different patients (n) are calculated. Asterisks depict significantly higher titers at $P < 0.05$.

adapted to replicate in CaCo-2 cells and further used to amplify its C-terminal nsP1a coding region and clone it within HAsV1 cDNA. Due to the low transfection efficiency described for CaCo-2 cell line, infectious RNA transcripts synthesized from every clone were first introduced into BHK-21 cells using the strategy described by Geigenmüller et al. (1997). Infectious viruses produced within BHK-21 cells were recovered in the presence of trypsin and were amplified by serial passages on CaCo-2 cells. These viruses thus corresponded to HVR-types IV, V, and VI, as well as XII which was obtained by direct transfection with the RNA produced from the original pAVIC.

Final mutant virus stocks were analyzed by sequencing approximately 90% of the replaced C-terminal nsP1a coding region. All viruses had introduced some nucleotide substitutions, which resulted in amino acid changes. In particular, in type IV mutant, residue Pro₆₆₁ was replaced by Leu₆₆₁. In type V, Ile₆₁₆ was replaced by Thr₆₁₆; Glu₇₇₂ was replaced by Asp₇₇₂; Gln₇₉₂ was replaced by His₇₉₂; and Ser₇₉₉ was replaced by Pro₇₉₉. In type VI, His₈₀₃ was replaced by Gln₈₀₃ (amino acid positions are numbered according to HAsV-1 Oxford reference strain accession no. L23513). Except those at positions 616 and 661, all mutations involved residues included in the HVR, which is prone to variability.

Comparison of virus productivity between different HAsV1 HVR-derived genotypes

In order to elucidate the specific effects of the genetic variability of the HVR on viral growth and RNA replication, CaCo-2 cells were infected with different HAsV-1 mutants

obtained (genotypes IV, V, and VI) using the original HAsV1 (genotype XII) as a control. Virus productivity and RNA replication properties were evaluated by measuring the titer of infectious virus in the supernatant of infected cells and by titrating the different types of RNA molecules extracted from intracellular membranes of infected cultures at 48 h pi (genomic RNA, antigenomic RNA, and subgenomic RNA). Primers from the ORF1a HVR were used to detect both the genomic and antigenomic RNA strands, while primers from ORF2 were used to detect the total positive-stranded RNA present within the cell, which includes both the genomic and subgenomic RNA molecules. The subgenomic positive RNA strands were calculated after subtracting genomic RNA titer from total RNA titer. Results of genomic RNA, antigenomic RNA, and subgenomic RNA titers are summarized in Fig. 2, as well as the infectious titer of viruses released to the supernatant of infected cells. Regarding genomic RNA, only the titer showed by type VI was significantly higher than that of type IV, while no differences were detected between the rest of genotypes. Two clear patterns were observed with respect subgenomic RNA. The one shared by genotypes IV and V, with higher titers, and the one of genotypes VI and XII, with significantly lower titers. Finally, the antigenomic titer of genotype V was significantly lower than that of genotypes VI and XII.

Alternatively, percentages of genomic, antigenomic, and subgenomic RNAs were also calculated, as shown in Table 1. HVR-derived genotype VI mutant exhibited a higher percentage of genomic RNA and a lower percentage of subgenomic RNA than genotypes IV and V. While for

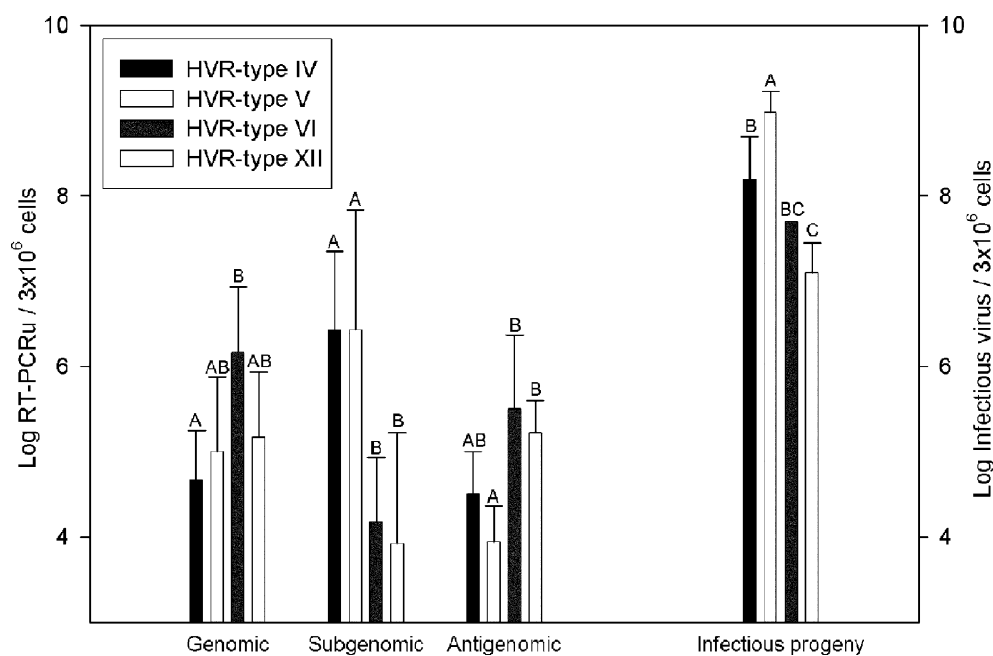


Fig. 2. Analysis of viral RNA titers found within CaCo-2 cells infected by different HAsV1 mutants belonging to HVR-types IV, V, VI, and XII. RNA titers were measured by end-point dilution RT-PCR and are expressed as the log of RT-PCR units per 3×10^6 cells. ANOVA comparisons between mutants were independently performed for each kind of RNA form. Common capital letters above bars indicate lack of statistical difference ($P < 0.05$). The values shown represent the average of three experiments, each titrated in duplicate.

Table 1
Percentages of cell-associated RNAs in different HVR type mutants

HVR type	Genomic RNA	Subgenomic RNA	Antigenomic RNA
IV	2.20 ± 1.50 ^A	95.71 ± 3.19 ^A	2.09 ± 1.69 ^A
V	13.58 ± 15.59 ^A	85.32 ± 17.24 ^A	1.10 ± 1.70 ^A
VI	80.30 ± 8.48 ^B	0.80 ± 0.08 ^B	18.90 ± 8.57 ^{AB}
XII	25.04 ± 21.63 ^A	17.34 ± 23.82 ^B	34.73 ± 26.24 ^B

Percentages of genomic RNA, subgenomic RNA, and antigenomic RNA were estimated from a total RNA population which was the sum of all three types of RNAs figured as RT-PCRu/3 × 10⁶ cells. Mean ± SD were calculated from three independent experiments, each titrated in duplicate. Common capital letters in superscript indicate lack of statistical difference ($P < 0.05$).

genotypes IV and V between 95.71% and 85.32% of the total positive-stranded RNA corresponded to subgenomic RNA, genotype VI produced essentially genomic RNA (80.30%) and hence limited amounts of subgenomic RNA. As a result, the titer of infectious progeny detected in the supernatant of genotype VI-infected cultures was significantly lower than that of genotype V and unexpectedly similar to that of genotype IV.

Thus, under identical conditions of infection and compared with types IV and V, type VI displayed different

replication properties. Type XII, employed in these studies as control, showed an intermediate replication pattern with regard to that of genotypes IV and V on the one hand and genotype VI on the other.

Proteomic characterization of mutants

Amino acid changes in the C-terminal nsP1a protein between the different HAsV1 mutants are shown in Fig. 3. Along the sequenced region, there were a total of 20 variable amino acid positions and 4 *indels*. All *indels* and most amino acid substitutions were located, as expected, within the HVR. According to physicochemical properties of amino acid substitutions, 45% of amino acid changes were conservative, 30% were semi-conservative, and 25% were non-conservative. Interestingly, some of these amino acid substitutions altered the number and position of the potentially phosphorylated sites of the HVR. Similarly, some of the detected substitutions affected the glycosylated sites. Comparisons among genotypes revealed that the serine phosphorylation sites included into the HVR showed a tendency to vary following a pattern, in which higher numbers of phosphoserine sites were associated with higher

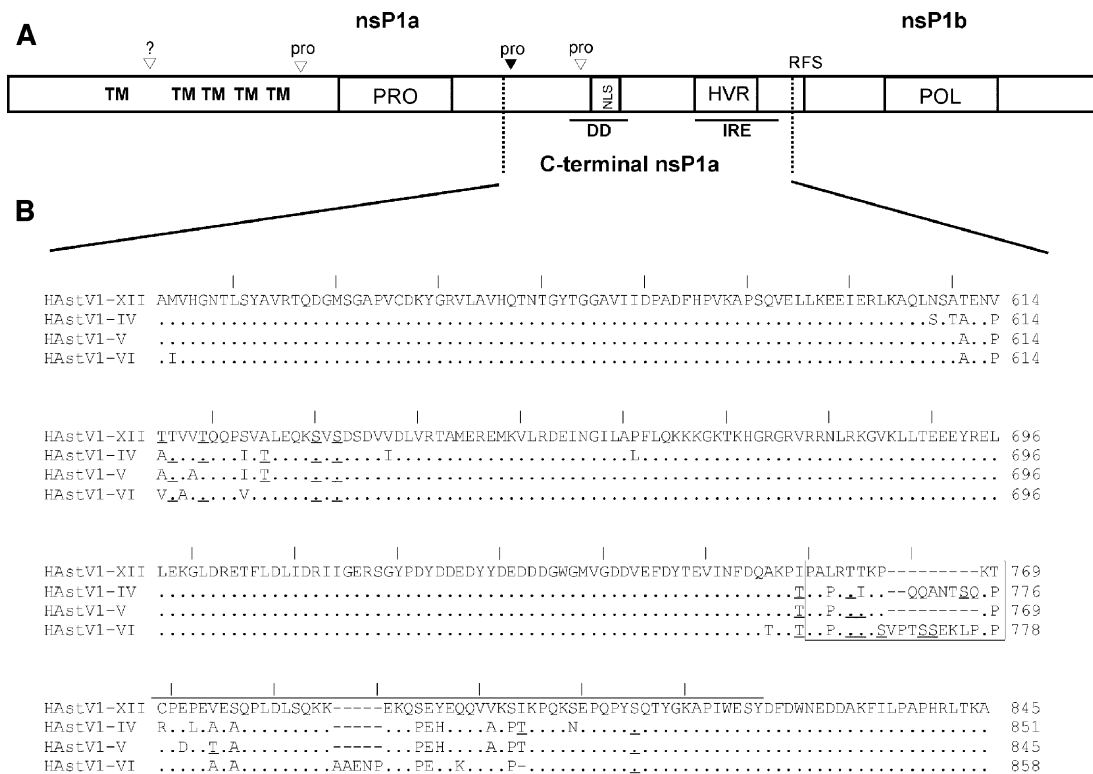


Fig. 3. Amino acid sequence analysis of the C-terminal nsP1a coding region replaced to generate the HAsV1 mutants. (A) Diagram of the nonstructural proteins encoded by HAsV ORF1a and ORF1b. The cleavage sites of nsP1a reported by Geigenmüller et al. (2002), Kiang and Matsui (2002), and Méndez et al. (2003) are indicated by black, open, and gray arrowheads, respectively. These cleavages seem to be dependent on both cellular proteases (?) and the viral protease (pro). Predicted transmembrane helices (TM), protease motif (PRO), predicted nuclear localization signal (NLS), death domain (DD), immunoreactive epitope (IRE), hypervariable region (HVR), and ribosomal frameshifting (RFS) between ORF1a and ORF1b are shown. (B) Sequence alignment of HAsV1 Oxford reference strain (type XII) and types IV, V, and VI mutants. Hypervariable region is boxed and sites liable to be post-translationally modified either by phosphorylation and/or O-glycosylation are shown in shadow and underlined, respectively. Amino acid positions are numbered according to HAsV1 Oxford reference strain (accession no. L23513).

and lower percentages of antigenomic and subgenomic RNAs, respectively (Table 2).

Although some extra point mutations appeared during the cloning procedure, as above stated, most of them were included in the HVR and none of them affected the phosphorylation and glycosylation sites.

Discussion

This work reports the effect of the genetic variability found within HAsV C-terminal nsP1a protein on viral growth and RNA replication. Different HVR-derived genotypes with distinct in-frame insertions and deletions have been established (manuscript in preparation), and three mutant viruses belonging to three out of these genotypes (types IV, V and VI) were constructed and assayed in comparison with the original type XII. Functional analysis of these mutants indicated that, compared to types IV and V, types VI and XII display a different behavior regarding viral RNA replication and viral growth. These differences in viral replication properties found between HAsV HVR-derived genotypes support the previously suggested role of this protein in modulating viral RNA replication (Guix et al., 2004b). Although the complete sequence of the recombinant viruses was not analyzed and hence the occurrence of mutation in other parts of the genome affecting the replication pattern cannot be completely ruled out, since no mutations were observed after sequencing a short fragment of the capsid (Noel et al., 1995), we assumed a low level of variation outside the HVR, as it can also be inferred after aligning the database sequences (data not shown). Within the same region, Willcocks et al. identified an in-frame 15-amino acid deletion related to the virus replication in certain cell lines other than CaCo-2 cells and not found in stool isolates (Willcocks et al., 1994). In the present study, this deletion was observed in both stool isolates and viruses adapted to CaCo-2 cells too, providing further evidence on the high variability of this region. Thus, this high degree of variability within the HVR and its association with different levels of RNA replication suggest a role of the C-terminal nsP1a protein in RNA replication, perhaps via interaction with viral and/or host-specific factors which could modulate the efficiency of RNA replication. The C-terminal nsP1a protein colocalizes with the endo-

plasmic reticulum-derived membranes and with viral RNA (Guix et al., 2004b). No differences in the localization of this protein have been observed between wild-type viruses belonging to different HVR-derived genotypes (data not shown), suggesting similar kinds of interactions.

Our results suggest the occurrence of two general patterns of modulation of RNA replication: on one hand the pattern shared by genotypes IV and V, and on the other the one showed by genotypes VI and XII. According to the accepted model, from the full-length genomic positive strand, the viral RNA polymerase copies a full-length negative antigenomic strand that acts as an intermediate for the final synthesis of the full-length genome and the subgenomic RNA (Lewis et al., 1994; Matsui et al., 2001; Monroe et al., 1991, 1993). Within this context, genotypes VI and XII, which synthesize lower numbers of subgenomic RNA and thus lower numbers of templates to produce structural proteins for encapsidation, should as well produce lower numbers of infectious progeny, as it is the case. On the contrary, genotypes IV and V, whose subgenomic RNA is as high as 85–95% of the total RNA, produce higher infectious progenies. However, the differences in infectivity titers between both patterns were not as high as one could expect from the subgenomic RNA levels. No explanation for this may be provided, not even after analyzing by ELISA the total structural proteins produced (data not shown). It might be hypothesized a higher capacity for cellular shut-off induction and thus an enhanced availability for the translation machinery that could compensate the lower subgenomic levels in genotypes VI and XII. Further experimental studies are required to provide more light in this issue.

Regulation of efficient minus and plus RNA strands, including the synthesis of a subgenomic RNA, is a complex process that involves many interactions of viral RNA with different virus and host proteins. Since the replication and transcription scheme postulated for astrovirus are inferred from the well-characterized life cycle of alphaviruses such as Sindbis virus and given the sequence similarity that has been observed between HAsV C-terminal nsP1a protein and alphavirus nsP3 (Guix et al., 2004b), functional parallelisms could be hypothesized between these two nonstructural proteins. Although the functions of the alphavirus nsP3 protein are not well defined, it has been shown to be extremely rich in insertions and deletions.

Table 2

Number of potentially phosphorylated or glycosylated residues in different HVR-derived genotypes distributed inside or outside the HVR

Genotype	Phosphorylation						Glycosylation			
	Inside HVR			Outside HVR			Inside HVR		Outside HVR	
	Tyr	Ser	Thr	Tyr	Ser	Thr	Ser	Thr	Ser	Thr
IV	0	2	1	5	3	0	2	2	2	4
V	0	2	2	5	3	0	1	3	2	3
VI	0	3	2	5	3	0	4	2	3	3
XII	0	4	2	5	3	0	0	0	2	2

Furthermore, a role in the regulation of the synthesis of both negative-strand RNA and subgenomic RNA has been suggested (LaStarza et al., 1994). In addition, it seems to be regulated by multiple Ser/Thr phosphorylation sites (Li et al., 1990). Moreover, mutations that affect some phosphorylation sites of alphavirus nsP3 significantly reduced the rate of viral RNA synthesis and exhibited greatly reduced pathogenicity in mice (Tuttila and Hinkkanen, 2003; Vihinen et al., 2001). Similar functional properties could be postulated for HAsV C-terminal nsP1a protein, since different patterns of serine phosphorylation sites within the HVR have been predicted for the studied astrovirus HVR-derived genotypes. Further investigations in the laboratory will be focused on these issues.

Interestingly, after quantifying the titer of viral genomes in stool specimens from children with gastroenteritis infected by distinct HVR-derived genotypes, differences in viral loads were found depending on the pattern of insertions and deletions. Consistently, in samples containing genotypes IV and V, a significantly higher viral titer was observed and, for genotype IV, which corresponded to serotype 3 strains, a correlation with persistent and severe gastroenteritis was also observed (Caballero et al., 2003). The differences in virus titer were not the result of the time after the onset of symptoms in which samples were collected, since it was randomly distributed among the genotypes, or of the total volume of feces, because no differences were observed, with the sole exception of feces with genotype IV that were shed in higher volumes and contained higher virus numbers per milliliter. Although the clinical significance of the higher viral titers in the feces of individuals infected with genotype IV is not fully understood, we hypothesize that it may be regulated by the C-terminal nsP1a protein.

Materials and methods

Cells and viruses

The human colon adenocarcinoma cell line CaCo-2 was grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and was used to propagate HAsV strains, as previously described (Pintó et al., 1994). Baby hamster kidney cells (BHK-21) were grown in MEM, supplemented with 10% FBS and 1 mM pyruvic acid sodium salt, and were used to transfect HAsV RNA and produce infectious viruses (Geigenmüller et al., 1997).

HAsV stool specimens and quantification of viral titer

A panel of 46 HAsV containing stool specimens from children with gastroenteritis was collected from an epidemiological study carried out in Spain (Guix et al., 2002), and was used to study the genetic variability. The number of

HAsV genomic RNA molecules per gram of feces was obtained by competitive RT-PCR using an internal standard, as previously described (Caballero et al., 2003).

Sequence analysis and genotype definition

Primers A1 (5'-CCTGCCCCGAGAACAACCAAGC-3', nucleotide positions 2363–2384 according to accession no. L23513) and A2 (5'-GTAAGATTCCCAGATTGGTGC-3', nucleotide positions 2534–2554 according to accession no. L23513) were used to amplify and sequence the HVR of the C-terminal nsP1a gene, as previously described (Guix et al., 2002; Willcocks et al., 1994). RT-PCR products of the expected size, ranging from 192 to 237 bp, were purified using a commercial kit (High Pure PCR Product Purification Kit, Roche) and were directly sequenced in an ABI Prism 377 automated DNA sequencer (Applied Biosystems), using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems). Sequence alignments were performed using CLUSTAL W (Higgins et al., 1994). In nucleotide alignments, positions of insertions and deletions (*indels*) were manually corrected according to their position in amino acid alignments. Nucleotide and amino acid distances were calculated as the number of substitutions per site (*p-dist*) using pairwise deletion for treating *indels* with the MEGA2.1 program (Kumar et al., 1993). According to genetic information provided by both *indels* and nucleotide distances, HAsV genetic types were defined so that all sequences within each type shared a common *indel* pattern, and so that nucleotide distances were lower than 7% within each type and higher than 7% between different types. Established HVR-derived genotypes were named with roman numerals. Based on the amino acid sequence, predicted post-translational modifications were also analyzed. Phosphorylation sites were predicted using the NetPhos 2.0 Server, considering a significance level of 0.9 (<http://www.cbs.dtu.dk/services/NetPhos/>) (Blom et al., 1999). O- β -GlcNAc O-linked glycosylation sites were predicted using the YinOYang 1.2 Server (<http://www.cbs.dtu.dk/services/YinOYang/>) (Gupta and Brunak, 2002).

Mutant viruses production

Mutagenesis of the C-terminal nsP1a gene coding region was performed using the HAsV1 cDNA clone pAVIC (HVR-type XII; kindly provided by S. M. Matsui, VA Palo Alto Health Care System, Palo Alto, USA; Geigenmüller et al., 1997). DNA cloning was carried out using established procedures (Sambrook et al., 1982). A region of 963 nucleotides (from nucleotide 1652 to 2614 of HAsV-1, accession no. L23513) was amplified from different wild type HAsV strains (types IV, V, and VI) using specific primers containing the sequence of the restriction sites for *Bgl*II and *Age*I enzymes (5'-AATGAAGATCTTGTGGTTTC-3' and 5'-AGTCAACCGGTGTGGCGCTG-3'; the

underlined indicate the restriction site introduced in each primer). The equivalent region of pAVIC plasmid was excised by digestion with the same pair of restriction enzymes. Recombinant pAVIC-IV, pAVIC-V, and pAVIC-VI plasmids were constructed by ligation of the restricted PCR fragments into *Bgl*II- and *Age*I-digested pAVIC. All resulting constructs were confirmed by DNA sequence analysis.

In vitro transcription of pAVIC, pAVIC-IV, pAVIC-V, and pAVIC-VI plasmids was performed using the T7 Cap Scribe kit (Roche), after linearization of 0.5 µg of each plasmid with the unique *Xho*I restriction site. Finally, infectious viruses were obtained after electroporation of 20 µl of transcribed RNA into BHK-21 cells using a Bio-Rad Gene Pulser. Briefly, subconfluent cell monolayers were trypsinized, washed with phosphate-buffered saline (PBS), and cooled on ice before being resuspended in ice-cold buffer (21 mM HEPES buffer [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, and 6 mM glucose). Subsequently, 2×10^6 cells were electroporated in a cuvette gap of 0.4 cm and a final volume of 0.8 ml, at 280 V and a capacitance of 250 µF; two pulses were used. Cells were diluted in 4 ml of 10% FBS cell growth medium and seeded on 35-mm dishes. After a 6-h incubation, 10% FBS medium was replaced by FBS-free medium. At 48 h post-transfection, trypsin type IX (Sigma) was added to a final concentration of 10 µg/ml, and cells and media were harvested after a 30-min incubation at 37 °C. Cells were lysed by three cycles of freeze/thawing, and the medium-cell lysate was used to passage the infectious viral progeny in CaCo-2 cells (type IV for pAVIC-IV, type V for pAVIC-V, and type VI for pAVIC-VI). The same procedure was employed to generate the progeny from the original pAVIC, which corresponds to type XII, to be used as control.

Viral RNA synthesis analysis

Genomic, antigenomic, and subgenomic RNA strands produced in CaCo-2 cells after infection with different recombinant viruses at a multiplicity of infection (moi) of 0.01 were titrated by end-point RT-PCR. At 48 h post-infection, 3×10^6 infected cells were disrupted in TN buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl) supplemented with 1% NP40, and after a 1-h incubation at 4 °C, cellular membranes were recovered by centrifugation at $7000 \times g$. The pellet was resuspended in 50 µl of TN buffer and processed for RNA extraction (Boom et al., 1990). Extracted RNA was titrated (5 µl) by end-point dilution RT-PCR. For genomic and antigenomic RNA titration, primers from the HVR were designed so that no mismatches were present between the target viral RNA and the primer: primers A1 and A2 for types V and XII; primers A1C (5'-CCTGCCCCGAGAACAATCAAGC-3') and A2C (5'-ATAAGATTCCCAGATTGGCGC-3') for type IV; and primers A1F (5'-CCTGCCCCGAGAACAACCAAT-3') and A2 for type VI. Either primer, forward (A1, A1C and

A1F) or reverse (A2, and A2C), were included in the RT step in order to detect both antigenomic RNA(–) and genomic RNA(+) strands, respectively. For total positive-stranded RNA titration (genomic and subgenomic RNA), we used primers corresponding to the ORF2, with some modifications (Noel et al., 1995) (Mon244 5'-GGTGTCA-CAGGACCAAAACC-3', and Mon245int 5'-CCTGAC-CACTAGTGGAAGA-3'), including primer Mon245int in the RT reaction. Each experiment was performed at least in duplicate. RNA titers were expressed as the log of RT-PCR units per 3×10^6 cells (RT-PCRu/ 3×10^6 cells).

Comparison of infectious virus productivity

Infectious viruses in the supernatant medium of infected cells (released virus) at 48 h pi after infecting with a moi of 0.01 were evaluated using a previously described method (Abad et al., 2001). Briefly, samples were pretreated with trypsin and 10-fold dilutions were inoculated onto CaCo-2 cell monolayers grown in 24-well plates. Trypsin was added to the post-infection medium, and the presence of virus in the supernatant of each dilution was evaluated by RT-PCR using specific primers derived from A1 and A2 for each corresponding virus, 6 days post-infection. All titration experiments were performed at least in duplicate.

Statistical analysis

Statistical comparisons between means from different groups were performed using the ANOVA analysis.

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